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The purpose of this study is to evaluate the role of the growth factor Cripto-1 (CR-1) in mammary development and carcinogenesis. This report describes the progress during the first year of support. During this period, it was discovered that the ribozyme which had been synthesized and tested acted through a primarily non-catalytic mechanism. This finding has been published (Appendix 1), where it was shown that this hammerhead-like molecule, targeted to the extreme 5' end of the CR-1 message, was very effective at eliminating CR-1 expression, even without catalytic activity. Due to this finding, we have designed 2 additional ribozymes and are making some changes in the expression vectors. It was also determined that developing mammary epithelium can be transduced by an injected retrovirus, but that expression of the transgene only occurs in the terminal end buds. Finally, it was found that overexpression of CR-1 in preneoplastic CL-S1 cells did not affect their growth. Work during the next year will include refinement of the ribozyme strategy and further analysis of *in vivo* transduction. In addition, we will conduct more experiments with altered CR-1 expression in the mammary cell lines to clarify the findings discussed in this report.

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Randall Kuntz 6/1/98

PI - Signature Date

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Introduction

The incidence rate for breast cancer generally increases with advancing age [1]. It remains unclear which variables may be involved in this relationship, though one candidate is a newly discovered member of the epidermal growth factor family, Cripto-1 (CR-1) [2-4]. Previous results from our laboratory indicate that CR-1 expression is increased in old mammary tissue [5], and that treatment of old mammary cells with tamoxifen *in vitro* reduces CR-1 mRNA levels (unpublished results). **These results suggest that CR-1 may be involved in carcinogenesis in older mammary tissue and also in the hormonal regulation of breast cancer.**

CR-1 is expressed at low levels in adult murine, spleen, heart, lung, brain, and breast [6, 7], and at elevated levels during fetal development [6] and during the growth of mammary epithelium during adolescence and pregnancy [7]. CR-1 is expressed in human breast cancer tissues [8, 9] and cell lines (both estrogen receptor positive and negative, [8]), as well as in colorectal tumors [10] and pancreatic cancer cells [11]. In two separate studies, 75-82% of breast cancer tissue samples were positive for CR-1, whereas 0-13% of adjacent, non-involved tissue samples were positive [8, 9], implying that CR-1 may be involved in growth regulation of breast cancer cells. In addition, over-expression of CR-1 in some immortalized murine mammary epithelial cells is sufficient to cause an increase in anchorage-independent growth, but not sufficient for tumor formation in nude mice [12]. Finally, treatment of CR-1-expressing teratoma cells with retinoic acid results in differentiation of the cells and shutoff of CR-1 transcription [2]. **All of the currently published data are correlative and provide only circumstantial evidence for a functional role of CR-1 in breast cancer.**

CR-1 plays a significant role in the neoplastic phenotype of some human colon cancer cell lines [13]. Treatment of CR-1-positive colon cancer cells with antisense RNA (either directly *in vitro* or by infection with a retroviral antisense expression vector) resulted in decreased CR-1 protein levels, reduction in both anchorage-dependent and -independent growth, and reduced ability to form tumors in nude mice [13]. **These results suggest that reduction, or better yet, elimination of CR-1 expression in CR-1-positive tumor cells may have a significant therapeutic effect.**

None of the previous studies have tested directly the role of CR-1 expression in the normal developing mammary gland. It is known that CR-1 is expressed in the adult gland, that expression is increased 2-3-fold during adolescent development of the epithelium and 20-fold during pregnancy and lactation, and that CR-1 protein can be found in the luminal epithelial cells, myoepithelial cells, and the cap cell population of the developing terminal end buds [7]. **Testing of the role of CR-1 in normal development may provide essential information for understanding the role of CR-1 in mammary carcinogenesis** and so is included as the *first technical objective* of this proposal. There is a current effort to generate CR-1 transgenic overexpression and null mutant mouse strains (pers. comm., N. Kenney, NIEHS), and we do not wish to duplicate this effort. Therefore, we will employ a strategy based on the generation of "transgenic tissue" as described by P.A.W. Edwards [14]. We plan to infect the normal progenitor epithelium *in situ* with a retrovirus expressing either CR-1 or a CR-1-specific ribozyme, and then evaluate the effects on development. This eliminates the step of culturing primary epithelium and infecting with the retrovirus *ex vivo*, and may eliminate any possible artifacts of the brief *in vitro* culture period. We have extensive experience with Dr. Edwards' tissue reconstitution model and have had preliminary success infecting progenitor mammary epithelium with a β-gal-expressing retrovirus (CA1) as demonstrated by staining whole-mounted mammary glands with x-gal.

The experiments described under the *second technical objective* of this proposal will demonstrate the effects of increased and decreased CR-1 expression on the tumorigenic characteristics of three related mammary epithelial cell lines. Plasmid vectors to be used for over- and under-expression (pECR1 and pRZECR-1, respectively) have been constructed and have proven to be effective in stably transfected

mammary epithelial cell lines (see Appendix 1 for an extensive description of the analysis of pRZECR-1). The retroviral over-expression vector (CA1CR) effectively increases CR-1 levels in mammary epithelial cell lines *in vitro*, and the retroviral under-expression vector (CA1CRZ) has been constructed, sequenced, and transfected into GP+E-86 retroviral packaging cells.

The *third and fourth technical objectives* bring the basic information learned about breast cancer etiology under the first two objectives closer to application for the patient. Current therapies for breast cancer have a variety of problems, including disfiguring surgery, systemic toxicity from radiation or chemotherapy, and various side effects experienced from tamoxifen therapy. Tamoxifen was tested in large-scale clinical trials as a particularly promising prophylactic agent which inhibits breast cancer development [15]. However, tamoxifen causes various replicative abnormalities in the liver, including liver neoplasms [16], and eventually, breast cancer in women treated with tamoxifen becomes resistant, probably by selection of resistant cell populations [17]. It would be advantageous to reproduce the benefits of tamoxifen, perhaps by directly reducing expression of genes which are inhibited after tamoxifen treatment, but without use of the agent itself. Since treatment of mammary epithelial cells with tamoxifen *in vitro* caused a reduction in CR-1 expression, decrease of CR-1 expression by other means may be a way to provide some of the benefit of tamoxifen without using the drug.

We propose the therapeutic use of a ribozyme expressed *in situ* for the following reasons. There are no known antagonists for the activity of CR-1 or its receptor (which has yet to be identified). Therefore, the two most likely methods for the specific reduction or elimination of CR-1 expression are antisense oligonucleotides and ribozymes. Both of these molecules have been used to alter the expression of specific genes *in situ* (see examples [13, 18-22]), and we chose to use a ribozyme rather than an antisense nucleotide because of the ribozyme's catalytic nature. Antisense oligonucleotides must generally be produced in stoichiometric amounts to eliminate synthesis of the target protein [23], while a single ribozyme molecule may cleave multiple copies of the target RNA [24, 25]. We have developed both a 39 nt CR-1-specific ribozyme and an antisense CR-1-expression construct consisting of the first 750 bp (including the entire coding region) of the murine CR-1 mRNA (see Appendix 1 for a complete description of the ribozyme). Preliminary studies in -SA mammary adenocarcinoma cells *in vitro* indicate that the ribozyme is much more effective at reducing CR-1 expression than is the antisense construct. CR-1 protein levels are reduced to below the level of detection with the ribozyme, while the antisense construct still shows some protein detectable on a western blot (data not shown). *In situ* expression (rather than exogenous administration of synthetic oligonucleotides) was chosen because we wish to perform experiments *in vivo* as well as *in vitro*, and synthesis of sufficient quantities of oligonucleotide to perform *in vivo* experiments is extremely expensive. If this technology is to be transferred into the clinical setting, such a treatment regimen would also be prohibitively expensive.

The use of non-replicative or attenuated viral vectors for the delivery of genes to cells is a well-established technology (for examples see [26-33]). Defective retroviral vectors have been employed in a number of human clinical trials as gene therapy vectors (review in [31]), and have been used experimentally for the transfer of transforming oncogenes into the rat mammary gland [27, 34].

This proposal should provide new information on the etiology of breast cancer, specifically concerning the role of an EGF family member (CR-1) in breast cancer and in normal mammary gland development. It will lay groundwork for the potential development of a novel class of therapeutics (gene therapy/anti-growth factors) for treatment of breast cancer, and it will contribute to the development of an animal model that should be valuable for refining the delivery methods of those novel therapeutics to the patient.

Hypotheses

1. Proper expression of Cripto-1 (CR-1) is required for the normal development of the mammary gland epithelial structure. (T.O. 1)
2. Overexpression of CR-1 will increase tumorigenicity of non-tumorigenic or moderately tumorigenic mammary epithelial cell lines. (T.O. 2)
3. Underexpression of CR-1 will decrease tumorigenicity of highly or moderately tumorigenic cell lines. (T.O. 2)
4. Efficient delivery of a ribozyme or other therapeutic gene to a developing tumor may be accomplished by injecting a retroviral vector at the tumor location. (T.O. 3)
5. Delivery of a retroviral vector containing a CR-1-specific ribozyme to an established CR-1-expressing tumor *in vivo* will result in the regression of that tumor. (T.O. 4)

Technical Objectives

1. Determine the role, if any, for CR-1 expression in the developing mouse mammary gland.
   a. Infect progenitor cells of the mammary epithelial tree with CR-1 over- or under-expressing retroviral vectors; examine mammary epithelial morphology.
   b. Quantify changes in CR-1 protein expression in the mammary gland.
2. Analyze the role of CR-1 in the neoplastic growth of murine mammary epithelial cell lines (CL-S1, -SA, and +SA) *in vitro* and *in vivo*.
   a. Look for changes in *in vitro* growth rate and anchorage-independent growth in cells over- and underexpressing CR-1.
   b. Examine tumor and metastasis production in syngeneic hosts *in vivo* in cells over- and underexpressing CR-1.
3. Establish a model system for the delivery of a gene to a mammary tumor using a retroviral vector.
   a. Use the defective (non-replicative) retroviral vector, CA1, which expresses β-galactosidase (β-gal), to infect tumor cells transplanted into epithelium-free mammary fat pads. Determine both the optimum conditions for infection of tumor cells and the largest tumor size which can be effectively infected.
4. Use a retroviral vector for treatment of mammary tumors *in vivo*.
   a. Use the retroviral ribozyme construct from T.O. 1 to treat mammary epithelial cell tumors. Generate mammary tumors and then use the optimum conditions determined in T.O. 3 for delivery of the ribozyme. Score alterations in tumor growth and the percentage of tumor and non-tumor cells infected.

Body

Results to date are indented from the previously submitted procedures and methods.

T.O. 1a. The CA1 retroviral vector (gift from P.A.W. Edwards, University of Cambridge) will be used for these experiments. The packaged vector will be produced in GP+E-86 cells, a mouse-specific retroviral packaging cell line [35]. Viral supernatants will be harvested, then filtered through 0.45 μm membranes to remove cells and cellular debris. If necessary, the virus may be concentrated by centrifugation through a 20% sucrose cushion at 34,000 x *g* for 6 hr, followed by resuspension in 1/100 of the original volume [27]. Viral titers will be determined by infection of +SA cells with serially diluted virus stocks, followed by staining with x-gal (or selection with G418). The number of blue cells (or

$G418^R$ colonies) will indicate the number of infectious virions per unit volume in the concentrated supernatant.

The epithelial ductal structure in the murine mammary gland does not develop until 3-6 weeks after birth. Therefore it is possible to produce epithelium-free mammary glands by removing the epithelial rudiment at 2-3 weeks of age [36]. It has been demonstrated that mammary epithelial cells transplanted into such a "cleared" gland will form a ductal structure, and that the mammary cells may be modified *in vitro* prior to implantation [14, 36]. Our results indicate that it is also possible to modify the epithelial rudiment itself by infection with a retrovirus.

Thirty mice (60 mammary glands) will be infected with either 2500 cfu (25 µl at 10^5 cfu/ml) of CA1 (β-gal only), CA1CR (expressing CR-1), or CA1CRZ (expressing a CR-1-specific ribozyme) in serum-free Dulbecco's modified Eagle's medium (DME) with 80 µg/ml polybrene; or treated with 25 µl DME plus 80 µg/ml polybrene alone. The infection will be performed by anaesthetizing the mouse, reflecting the skin to expose the #4 (inguinal) mammary gland, and injecting a 25 µl virus suspension into the gland. The wound will be closed with surgical clips, and aseptic technique will be maintained throughout as recommended by the WSU IACUC Guidelines for Survival Rodent Surgery. The use of both #4 glands will allow us to directly compare two treatments in the same mouse, any quantitative data may then be compared using a paired T-test; otherwise quantitative comparisons will be made among all groups using ANOVA and Fisher's PLSD. The initial chosen pairings will be CA1 with CA1CR and CA1CRZ with DME. If other specific pairings are warranted by the findings, the experiment can be repeated.

Three mice from each pairing (thus 3 glands from each treatment) will be sacrificed after 1 week, and every week thereafter for a total of 5 weeks. This schedule will allow us to follow the development of the mammary epithelium and evaluate the effect of the treatments throughout the process of gland development. In the future, it may be informative to examine the morphology of transduced glands in pregnant or aged mice, due to the higher observed levels of CR-1 expression in those glands. Changes in gland morphology may be qualitatively assessed by observation of the whole mounts. If desired, glands can be dissolved out of Permount with xylene and then embedded in paraffin for sectioning. Changes in CR-1 expression can be assessed in these sections by immunohistochemistry using a CR-1-specific antibody we have prepared against a synthetic peptide (first described in [10]).

The following experiments have been performed; these pertain to Tasks 1, 2, and 3 of the Statement of Work. Viral producer cells (GPE) containing the CA1 vector or the CA1 vector with CR-1 were injected (250,000 per mammary gland) into the glands of 3 week old mice. Injections were performed both adjacent and distal to the nipple end. We observed tumors in the CA1+Cripto (CA1CR) glands in 2/6 mice. None showed tumors in the CA1 only side. We observed transduction of the developing epithelium, though this was more difficult to observe in the animals which developed tumors. We also implanted beads (Cytoline 1, Pharmacia) loaded with the same cells. These glands did not develop tumors in 4/4 mice, and the producer cells appeared to stay confined to the bead rather than growing out into the gland. Transduction of growing epithelium was definitely observed in these glands. It is unclear at this time if the production of tumors by the producer cells in this small number of animals is significant.

In addition to the producer line experiments, we injected the glands with purified recombinant retrovirus, as described in the proposed method (n=10, sacrificing 2 mice per week instead of 3). We observed transduction of the epithelium and no tumors, however, the transduction was restricted to the actively growing portions of the ducts (the end buds). This was the same result observed for the animals that received the producer lines confined to the Cytoline beads. The lack of staining along the entire duct

may indicate that the viral promoter is shut off in these non-dividing epithelial cells. The glands receiving the CA1CR vector appeared to show an increased rate of duct growth, though the low number of repetitions at this point bring the significance of this observation somewhat into question.

Due to the final characterization of the CR-1-specific ribozyme as being non-catalytic (see Appendix 1), we have not started those *in vivo* experiments yet (CA1CRZ vector). We feel that it may be important to have catalytic activity to maximize CR-1 elimination in a situation where we are not using cloned cell lines, so we are developing 2 new ribozymes and putting them in a self-splicing vector to maximize the chance of generating a catalytic molecule.

T.O. 1b. For precise quantification of CR-1 protein levels, the experiment will be repeated, and glands will be harvested for protein analysis by western blot. CR-1 will be detected using the same antibody as for immunohistochemistry.

No quantification of CR-1 levels has been performed yet in these glands (SOW Task 4).

T.O. 2 We have designed and tested a hammerhead ribozyme [20, 37] that recognizes nucleotides 12-28 of the murine CR-1 mRNA and cuts after the GUC triplet at nucleotides 18-20 (See Appendix 1). A search of the GenBank database (FASTA, GCG Wisconsin Package) revealed no significant nucleotide sequence identity to any other published sequence, including related EGF family members.

The first task that we set about was to finish characterization of the CR-1 specific ribozyme and to publish that information. During the course of this effort it was discovered that the ribozyme was very effective at eliminating CR-1 expression in clones transfected with an expression plasmid (as reported in the original proposal), but that it was not accomplishing this by cleaving the mRNA. Northern blots were deemed inconclusive, as there is not a great deal of CR-1 message made in -SA cells, and the size is similar to that of 18S rRNA. We then used RT-PCR, with primers that span the putative cut site. It was found that the message was not being cleaved in most clones that showed elimination of protein expression. Therefore, it appeared that the ribozyme was quite effective, but perhaps due to its site of attack rather than catalytic activity. Of significance, we believe that this was the first report of using a ribozyme to attack CR-1 and this was also the first report of targeting one to the extreme 5' end of the message. This target may be so close to the 5' cap that it blocks 40S ribosomal subunit binding, as has been shown for some hairpin structures in that region of a message [38].

It is unclear why the ribozyme is not catalytic. Perhaps it is due to poor folding due to the long regions 5' and 3' of the ribozyme domain in the ribozyme transcript. During the course of investigation of the mechanism of ribozyme activity, we were able to obtain a vector for the expression of ribozymes which contains self-cleaving ribozymes which flank the ribozyme of interest 5' and 3'. This construct should allow us to express the ribozyme without the long regions of "useless" RNA adjacent to the ribozyme, perhaps enhancing proper folding and catalytic activity. We have also designed two more ribozymes to test, another targeting the 5' untranslated region of the message, and one targeting the coding region. We are currently in the process of constructing the expression vectors for these ribozymes

The CL-S1 cell line was derived from a preneoplastic BALB/c mammary nodule, and the -SA, and +SA target cell lines were isolated from a spontaneous adenocarcinoma derived from the same nodule ([39]). CL-S1 cells are anchorage-dependent and do not form tumors in syngeneic hosts; -SA cells do not form colonies efficiently in soft-agar, but will form tumors in syngeneic hosts after injection *in vivo*; +SA cells display efficient colony formation *in vitro*, and are neoplastic and metastatic (pulmonary metastases after intravenous injection) when inoculated into syngeneic hosts *in vivo* [39]. CR-1 expression is strong in +SA cells, slightly lower in -SA cells, and low in CL-S1 cells (unpublished results). A CR-1 over-expression retroviral vector has also been constructed (CA1CR) and used to infect CL-S1.

T.O. 2a. Changes in the growth phenotype of the transfected clones which show the greatest difference in CR-1 levels from the parental line will be evaluated *in vitro* by examining growth rate and the relative efficiency of colony formation in soft agar [39] in triplicate cultures of the various transfected lines. Differences in amount of cellular DNA and colony numbers in soft agar will be analyzed using ANOVA and Fisher's PLSD.

Progress on SOW task 7: Growth experiments indicate that overexpression of CR-1 is not sufficient to allow CL-S1 cells to grow in soft agar (either transfected with pECR1 or transduced with CA1CR), nor do they show increased anchorage-dependent growth rate. Transfection of -SA cells with pRZECR-1 did result in elimination of CR-1 expression, but no change in growth rate or colony formation was observed.

T.O. 2b. The clones analyzed in T.O. 2a will be implanted into syngeneic mice for *in vivo* analysis. Five x 10^5 cells will be injected into epithelium-free (cleared) mammary fat pads of 6-8 week old female BALB/c mice. Six to eight week old animals will be used because (1) the fat pads are larger in these animals, so the surgical implantations are mechanically easier to perform; and (2) at three weeks of age, the mice are still in puberty, and we wish to minimize possible effects of the hormonal state of the animal on the results. The mice will be palpated twice per week for tumor development and tumor size will be measured with a caliper. In untreated mice, +SA-derived tumors are typically palpable after 14-21 days [39]. Therefore, mice will be sacrificed at the end of 4 weeks, unless they are moribund prior to that time, in which case they will be sacrificed immediately and the date of sacrifice noted. Mammary tumors will be further evaluated after fixation, clearing and staining of whole mammary glands. Both whole mounts and sections from them will be analyzed with respect to outgrowth pattern of the transplanted cells (normal vs. hyperplastic). Differences in tumor size (and number if separate tumors are generated) will be compared among all cell types by ANOVA and Fisher's PLSD.

The same cell populations (1 x 10^5 cells) will be injected into the tail vein of 6-8 week old BALB/c mice to evaluate the potential for the formation of pulmonary metastases [39]. Three mice will be used for each of the cell populations, and they will be sacrificed at 4 weeks after inoculation. Differences in pulmonary tumor number and size will be analyzed using ANOVA and Fisher's PLSD.

The following experiments pertain to SOW Task 8. Injection of CL-S1 cells transduced with the CA1 and CA1CR vectors into mice gave the following results. Fourteen glands were injected with 500,000 cells of the CL-S1/CA1CR cells, resulting in 1 small tumor. Eight glands were injected with the CL-S1/CA1 cells, and no tumors were observed. Four glands each were injected with 1,000,000 cells of either CL-S1/CA1CR or CL-S1/CA1; no tumors were observed. In addition, 26 glands were implanted with the CL-S1/CA1CR cells on Cytoline beads, and no tumors were observed. From these results, we do not believe that overexpression of CR-1 is sufficient to cause transformation of the immortal CL-S1 line. No metastasis

experiments were performed, as this is a more stringent test than simple tumor development in the gland.

The most striking observation from the injection experiments with the CL-S1 cells is that they appear to "home" to the ducts of an intact mammary gland. In a cleared gland, they appear as a diffuse blue staining throughout the gland, but injection in an intact gland results in intense blue staining associated with the ducts and alveoli. It is not clear why this occurred, but the phenomenon was absolutely reproducible.

Implantation of these -SA/RZECR-1 cells into syngeneic mice resulted in formation of tumors in 11/26 mice when the -SA/BKCMV control was present on the contralateral side. Tumors developed in 0/5 when -SA/RZECR-1 cells were implanted alone. Several of the tumors were excised from the mice, and all displayed strong CR-1 expression. It was unclear whether the tumors were derived from recruited -SA/BKCMV cells or if the ribozyme was no longer functional in the -SA/RZECR-1 cells. At this point it is still premature to say that elimination of CR-1 expression reduces tumor formation *in vivo*.

T.O. 3a. Five x 10^5 +SA cells will be implanted into the cleared #4 fat pads of sixteen 6-8 week old mice. The position of the +SA cells in the gland will be marked on the skin surface to facilitate later injection of viral suspensions. The glands will be infected with 2 x 10^6 cfu (in 2 injections of 25 µl at 4 x 10^4 cfu/µl) of the β-gal-expressing CA1 retroviral vector in the presence of 80 µg/ml polybrene [27]. Injections will begin from 0-14 days after the implantation of the tumor cells and will be repeated every other day until day 18 (see Table 3 for a summary). All mice will be sacrificed on day 28 after implantation of tumor cells and the glands will be removed for analysis. Glands will be fixed and stained for β-gal. Tumor size, percentage of infected (β-gal positive) tumor cells, percentage of infected surrounding cells, and distance of viral spread from the injection point will be recorded. The optimal time after implantation of the tumor cells and the maximum time after implantation (and therefore maximum tumor size) allowing efficient infection with the vector will be determined. The planned viral inoculum will result in a vector: target ratio of 4:1 at the time of tumor cell implantation, if all of the transplanted tumor cells survive. Should these vector:target ratios prove insufficient for efficient infection of the tumor, we can either reduce the initial tumor cell dose or attempt to further increase the viral titer.

SOW Tasks 9 and 10; these experiments have not yet begun.

T.O. 4a. The retroviral constructs CA1 and CA1CRZ used in T.O. 1 will be used to treat +SA tumors *in vivo*. +SA tumors will be generated in the cleared #4 mammary fat pads of fifteen 6-8 week old mice as described in T.O. 3. Ten of the mice will be infected with CA1 and CA1CRZ at the optimal conditions determined under T.O. 3 (CA1 on one side CA1CRZ on the other). The five remaining mice will receive only DME containing 80 µg/ml polybrene (in both sides) as a control (see Table 4 for a summary). This design will allow a paired comparison (controlling for host variation) between CA1 and CA1CRZ, and will allow a general comparison (ANOVA + Fisher's PLSD) among all three groups. Glands will be scored for tumor size, percentage of infected tumor cells, and percentage of infected surrounding cells (determined by β-gal staining). A group size of ten (glands) for each treatment is used here rather than the six used in T.O. 2, because reliance on infection of the tumor cells rather than using a stably transfected tumor cell line may increase experimental variability.

SOW Task 11; these experiments have not yet begun.

Conclusions

1. It is possible to eliminate CR-1 protein expression through the use of a ribozyme-like molecule, even without catalytic activity. This decrease is substantially better than that previously observed with antisense strategies [13]. This may indicate that RNA molecules with strong secondary structure can be targeted to the extreme 5' end of a message to obtain excellent reduction or elimination of expression.

2. Overexpression of Cripto-1 in the preneoplastic line CL-S1 did not cause an increase in growth, colony formation in soft agar, or tumor formation *in vivo*.

3. Elimination of CR-1 expression in -SA cells did not affect growth rate or colony formation *in vitro*. The *in vivo* results are not yet clear.

4. It is possible to transduce nascent mammary epithelium with injection of either retrovirus or retrovirus-producing cells. However, expression appears to be restricted to actively dividing cells, not the entire epithelial tree.

References

1. Kessler L.G. 1992. The relationship between age and incidence of breast cancer. *Cancer* **69:**1896-1903.

2. Ciccodicola A., Dono R., Obici S., Simeone A., Zollo M., and Persico M.G. 1989. Molecular characterization of a gene of the 'EGF family' expressed in undifferentiated human NTERA2 teratocarcinoma cells. *EMBO J.* **8:**1987-1991.

3. Dono R., Montuori N., Rocchi M., De Ponti-Zilli L., Ciccodicola A., and Persico M.G. 1991. Isolation and characterization of the cripto autosomal gene and its x-linked related sequence. *Am. J. Hum. Genet.* **49:**555-565.

4. Saccone S., Rapisarda A., Motta S., Dono R., Persico M.G., and Della Valle G. 1995. Regional localization of the human EGF-like growth factor cripto gene (TDGF-1) to chromosome 3p21. *Hum. Genet.* **95:**229-230.

5. Herrington G., Ram T., Mills R., and Hosick H. 1994. Expression of EGF-related growth factors in mouse mammary gland of various ages. *J. Cell. Biochem. Suppl.* **18D:**231.

6. Dono R., Scalera L., Pacifico F., Acampora D., Persico M.G., and Simeone A. 1993. The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. *Development* **118:**1157-1168.

7. Kenney N.J., Huang R.-P., Johnson G.R., Wu J.-X., Okamura D., Matheny W., Kordon E., Gullick W.J., Plowman G., Smith G.H., Salomon D.S., and Adamson E.D. 1995. Detection and location of amphiregulin and cripto-1 expression in the developing postnatal mouse mammary gland. *Mol. Reprod. Dev.* **41:**277-286.

8. Normanno N., Qi C.-F., Gullick W.J., Persico G., Yarden Y., Wen D., Plowman G., Kenney N., Johnson G., Kim N., Brandt R., Martinez-Lacaci I., Dickson R.B., and Salomon D.S. 1993. Expression of amphiregulin, cripto-1, and heregulin a in human breast cancer cells. *Int. J. Oncol.* **2:**903-911.

9. Qi C.-F., Liscia D.S., Normanno N., Merlo G., Johnson G.R., Gullick W.J., Ciardiello F., Saeki T., Brandt R., Kim N., Kenney N., and Salomon D.S. 1994. Expression of transforming growth factor α, amphiregulin, and cripto-1 in human breast carcinomas. *Br. J. Cancer* **69:**903-910.

10. Saeki T., Stromberg K., Qi C.-F., Gullick W.J., Tahara E., Normanno N., Ciardiello F., Kenney N., Johnson G.R., and Salomon D.S. 1992. Differential immunohistochemical detection of amphiregulin and cripto in human normal colon and colorectal tumors. *Cancer Res.* **52:**3467-3473.

11. Friess H., Yamanaka Y., Büchler M., Kobrin M.S., Tahara E., and Korc M. 1994. Cripto, a member of the epidermal growth factor family, is over-expressed in human pancreatic cancer and chronic pancreatitis. *Int. J. Cancer* **56:**668-674.

12. Ciardiello F., Dono R., Kim N., Persico M.G., and Salomon D.S. 1991. Expression of cripto, a novel gene of the epidermal growth factor gene family, leads to in vitro transformation of a normal mouse mammary epithelial cell line. *Cancer Res.* **51:**1051-1054.

13. Ciardiello F., Tortora G., Bianco C., Selvam M.P., Basolo F., Fontanini G., Pacifico F., Normanno N., Brandt R., Persico M.G., Salomon D.S., and Bianco A.R. 1994. Inhibition of CRIPTO expression and tumorigenicity in human colon cancer cells by antisense RNA and oligodeoxynucleotides. *Oncogene* **9:**291-298.

14. Edwards P.A.W. 1993. Tissue reconstitution models of breast cancer. *Cancer Surveys* **16:**79-96.

15. Love R.R. 1992. Tamoxifen prophylaxis in breast cancer. *Oncology* **6:**33-43.

16. Sargent L.M., Dragan Y.P., Bahnub N., Wiley J.E., Sattler C.A., Schroeder P., Sattler G.L., Jordan V.C., and Pitot H.C. 1994. Tamoxifen induces hepatic aneuploidy and mitotic spindle disruption after a single in vivo administration to female Sprague-Dawley rats. *Cancer Res.* **54:**3357-3360.

17. Graham II M.L., Smith J.A., Jewett P.B., and Horwitz K.B. 1992. Heterogeneity of progesterone receptor content and remodeling by tamoxifen characterize subpopulations of cultured human breast cancer cells: analysis by quantitative dual parameter flow cytometry. *Cancer Res.* **52:**593-602.

18. Ciardiello F., Bianco C., Normanno N., Baldassarre G., Pepe S., Tortora G., Bianco A.R., and Salomon D.S. 1993. Infection with a transforming growth factor a anti-sense retroviral expression vector reduces the in vitro growth and transformation of a human colon cancer cell line. *Int. J. Cancer* **54:**952-958.

19. Kashani-Sabet M., Funato T., Florenes V.A., Fodstad O., and Scanlon K.J. 1994. Suppression of the neoplastic phenotype in vivo by an anti-ras ribozyme. *Cancer Res.* **54:**900-902.

20. Sullivan S.M. 1994. Development of ribozymes for gene therapy. *J. Invest. Dermatol.* **103:**85S-89S.

21. Shore S.K., Nabissa P.M., and Reddy E.P. 1993. Ribozyme-mediated cleavage of the BCRABL oncogene transcript: in vitro cleavage of RNA and in vivo loss of P210 protein-kinase activity. *Oncogene* **8:**3183-3188.

22. Moroni M.C., Willingham M.C., and Beguinot L. 1992. EGF-R antisense RNA blocks expression of the epidermal growth factor receptor and suppresses the transforming phenotype of a human carcinoma cell line. *J. Biol. Chem.* **267:**2714-2722.

23. Denhardt D.T. 1992. Mechanism of action of antisense RNA: sometime inhibition of transcription, processing, transport, or translation. *Ann. N. Y. Acad. Sci.* **660:**70-76.

24. Cech T.R. and Uhlenbeck O.C. 1994. Hammerhead nailed down. *Nature* **372:**39-40.

25. Uhlenbeck O.C. 1987. A small catalytic oligoribonucleotide. *Nature* **328:**596-600.

26. Boris-Lawrie K. and Temin H.M. 1994. The retroviral vector - Replication cycle and safety considerations for retrovirus-mediated gene therapy. *Ann. N. Y. Acad. Sci.* **716:**59-71.

27. Wang B., Kennan W.S., Yasukawa-Barnes J., Lindstrom M.J., and Gould M.N. 1991. Carcinoma induction following direct in situ transfer of v-Ha-ras into rat mammary epithelial cells using replication-defective retrovirus vectors. *Cancer Res.* **51:**2642-2648.

28. Carter B.J. 1992. Adeno-associated virus vectors. *Curr. Opin. Biotechnol.* **3:**533-539.

29. Latchman D.S. 1994. Herpes simplex virus vectors for gene therapy. *Mol. Biotechnol.* **2:**179-195.

30. Kremer E.J. and Perricaudet M. 1995. Adenovirus and adeno-associated virus mediated gene transfer. *Br. Med. Bull.* **51:**31-44.

31. Vile R.G. and Russell S.J. 1995. Retroviruses as vectors. *Br. Med. Bull.* **51:**12-30.

32. Pepose J.S. and Leib D.A. 1994. Herpes simplex viral vectors for therapeutic gene delivery to ocular tissues. *Invest. Ophthalmol. Vis. Sci.* **35:**2662-2666.

33. Georges R.N., Mukhopadhyay T., Zhang Y., Yen N., and Roth J.A. 1993. Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct. *Cancer Res.* **53:**1743-1746.

34. Wang B., Kennan W.S., Yasukawa-Barnes J., Lindstrom M.J., and Gould M.N. 1991. Frequent induction of mammary carcinomas following neu oncogene transfer into in situ mammary epithelial cells of susceptible and resistant rat strains. *Cancer Res.* **51:**5649-5654.

35. Markowitz D., Goff S., and Bank A. 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virology.* **62:**1120-1124.

36. DeOme K.B., Faulkin L.J., Bern H.A., and Blair P.B. 1959. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.* **19:**515-520.

37. Forster A.C. and Symons R.H. 1987. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* **49:**211-220.

38. Kozak M. 1989. Circumstances and Mechanisms of Inhibition of Translation by Secondary Structure in Eukaryotic mRNAs. *Mol. Cell. Biol.* **9:**5134-5142.

39. Danielson K.G., Anderson L.W., and Hosick H.L. 1980. Selection and characterization in culture of mammary tumor cells with distinctive growth properties in vivo. *Cancer Res.* **40:**1812-1819.

Appendix 1

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 245, 774–779 (1998)
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Reduction of Cripto-1 Expression by a Hammerhead-Shaped RNA Molecule Results from Inhibition of Translation Rather Than mRNA Cleavage

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Cripto-1 (CR-1) is a transforming growth factor which has been associated with breast, colon, and pancreatic cancer. Overexpression of CR-1 in non-tumorigenic mouse mammary epithelial cells and fibroblasts results in an increase in anchorage-dependent and -independent growth *in vitro*. Reduction of CR-1 expression in human colon carcinoma or embryonal teratoma cells results in a decrease in growth *in vitro*. In an effort to better define the role of CR-1 in breast cancer, we have developed an underexpression vector for CR-1 to reduce CR-1 levels in a tumorigenic mouse mammary epithelial cell line (-SA). This vector specifically targets the expression of the murine homolog of CR-1 in murine cancer lines and utilizes a hammerhead ribozyme-like structure directed toward the extreme 5′ end of the Cripto-1 mRNA. We dramatically reduced expression of CR-1 through the expression of this RNA. This is the first use of a ribozyme-like molecule to alter Cripto-1 expression. This ribozyme-shaped molecule appears to act principally through a block in translation. A possible mechanism for this block is described, and its implications for modifying expression of other bioactive proteins are discussed.

Cripto-1 (CR-1) is a member of the Epidermal Growth Factor (EGF) family. It was originally cloned from human embryonal carcinoma cell line NT2D1 (1), and the murine homolog was later cloned from mouse embryos (2). CR-1 is expressed in 60-80% of colorectal cancers, but in only 0-7% of normal colon mucosal cells (3,4). CR-1 can be detected in several human breast cancer cell lines (3,4), and has been detected in 75-82% of human breast tumors, with none of the protein identified in adjacent, non-cancerous tissue (3-5). CR-1 has also been found in gastric carcinomas (6) and pancreatic cancer (7).

In addition to the descriptive studies cited above, CR-1 expression has been experimentally altered in certain cell lines, and the results demonstrate that it acts as a transforming growth factor. Overexpression of human CR-1 in immortalized, non-tumorigenic mouse fibroblasts, NIH3T3, resulted in a pronounced increase in anchorage-independent growth *in vitro* (1). Overexpression of the human CR-1 gene in the immortalized, non-tumorigenic mouse mammary epithelial line, NOG-8, resulted in increased anchorage-dependent and -independent growth *in vitro,* but not in tumorigenicity *in vivo* (8). Finally, incubation of two human mammary carcinoma lines and one nontransformed mammary epithelial cell line with synthetic peptides derived from the CR-1 sequence or with conditioned medium from CHO cells transfected with the human CR-1 gene resulted in increased anchorage-dependent growth *in vitro* (9).

In addition to these overexpression studies, responses to reduction of CR-1 expression through the use of antisense RNA oligonucleotides or antisense expression vectors have also been assessed. In human colon carcinoma cells (GEO), reduction of CR-1 expression inhibited anchorage-dependent and -independent growth *in vitro* and reduced tumorigenicity *in vivo* (10). Also, reduction of CR-1 expression in NT2D1 embryonal carcinoma cells resulted in decreased anchorage-dependent and -independent growth *in vitro* (11).

Although these expression reduction studies paint a clear picture of the importance of CR-1 expression in colon cancer cells and embryonic teratoma cells, the data for breast cancer cells are less clear. To gain further insight into the potential role for CR-1 in mammary tumorigenesis, we have chosen to examine the effect of reducing CR-1 expression in a tumorigenic mouse mammary epithelial cell line, -SA. This cell line,

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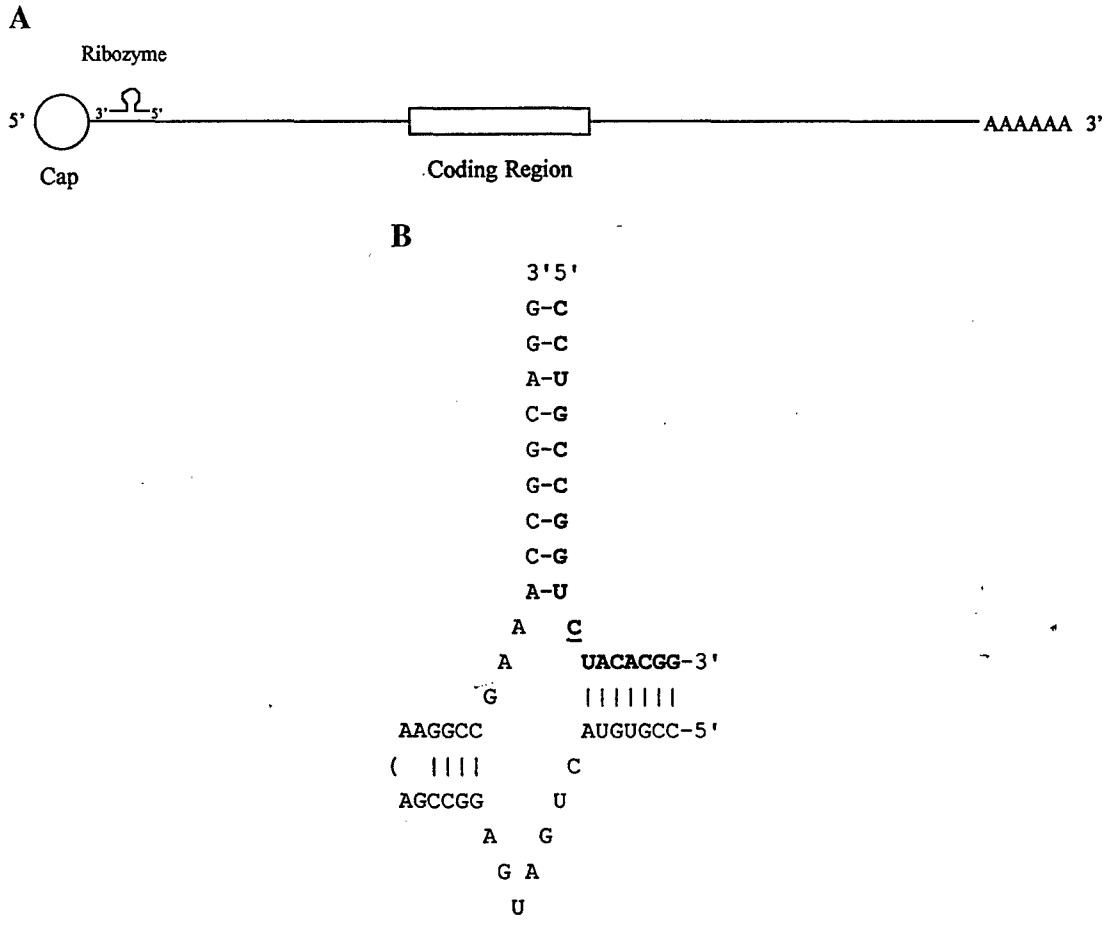


FIG. 1. CR-1-specific hammerhead ribozyme. (A) Diagram of the processed CR-1 mRNA, showing the ribozyme recognition sequence with respect to the 5′ cap, coding region, and polyA tail. (B) Sequence of the ribozyme and its presumed binding conformation to the CR-1 message.

which was isolated from a spontaneous mouse mammary adenocarcinoma, does not form colonies in soft agar, but does form tumors *in vivo* (12).

Finally, these experiments focus on providing an effective way to decrease murine CR-1 expression in mouse cells. Though the human and murine genes exhibit 80% homology overall (2), it will be necessary to carefully examine the effects of altering CR-1 expression using protein and cells from the same species, rather than simply using the human CR-1 protein in all cells, as has been done to date.

MATERIALS AND METHODS

Plasmids and constructs. The Cripto-1-specific hammerhead molecule was synthesized as two complementary single-stranded DNAs, 43 nucleotides long (Eppendorf Custom Oligonucleotides, Madison, WI). Two μg of each oligo were mixed and heated to 75°C for 10 minutes. They were allowed to cool slowly to 4°C and then electrophoresed on a 3% Metaphor (FMC, Rockland, ME) agarose gel to verify annealing. The annealed molecule was then ready for cloning into an expression vector.

The basic expression construct used in this study was pBKCMV (Stratagene, La Jolla, CA). This is a eukaryotic expression vector which expresses the *neo* gene from an SV40 promoter to allow for selection of stable transfectants, and the cloned sequence from the cytomegalovirus (CMV) immediate early promoter to provide high levels of expression. The hammerhead construct, pRZECR-1, was made by cloning the Cripto-1-specific ribozyme sequence between the *Bam*HI and *Eco*RI sites in pBKCMV. This puts the hammerhead sequence under the control of the strong CMV promoter. Correct insertion was established by restriction enzyme analysis and verified by sequencing (Applied Biosystems automated sequencer, Laboratory for Biotechnology and Bioanalysis, WSU, Pullman, WA).

Cells and cell culture. The -SA cell line was isolated from a spontaneous adenocarcinoma that developed within a preneoplastic mouse mammary nodule (12). This line does not form colonies in soft agar, does form tumors in syngeneic hosts after injection *in vivo,* and is not metastatic. Unless otherwise indicated, all cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% bovine calf serum (Hyclone, Ogden, UT). Cells were maintained at 37°C in a 5% CO_2 environment.

Transfection of pBKCMV (control) and pRZECR-1 into mammary tumor cells was accomplished using Lipofectamine (Gibco/BRL, Gaithersburg, MD), according to the manufacturer's instructions. Two to ten μg of plasmid was used with 10 μl of Lipofectamine for each 35 mm dish of cells. Transfections were performed in the absence of serum (Optimem medium, Gibco/BRL). Transfected cells were refed with DMEM supplemented with 10% bovine calf serum after 24 hr. Stable transfectants were selected, beginning 72 hr after the transfection, by growth in the presence of 800 μg/ml Geneticin

(G418, Gibco/BRL). At least 12 G418-resistant clones were isolated from each transfection of -SA cells for evaluation of mRNA and protein production.

Production of the CR-1 antiserum (CR-67). A polyclonal anti-CR-1 antiserum was made by immunizing rabbits with a synthetic peptide (CPPSFYGRNCEHDVRKE) which has been demonstrated to elicit effective CR-1 specific antibodies (4); this antibody is later called anti-CR-67 (9). This peptide is 100% conserved between human and murine CR-1 (2). The polyclonal antiserum was produced by Quality Controlled Biochemicals, Inc., Hopkinton, MA. The peptide was synthesized, purified, conjugated to KLH, and injected into rabbits. Animals were boosted and bled, and the activity of the serum was verified by ELISA against the peptide.

Western blotting. Cells were grown to 90% confluence in 10 cm tissue culture dishes, rinsed with PBS (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), then lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 7.5) at 4°C. Samples were sonicated on ice and frozen at −20°C. Identical cell equivalents (roughly 300,000 cells) of lysates were diluted 1:1 with 2× Laemmli loading buffer (100 mM Tris, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol), supplemented with β-mercaptoethanol to a final concentration of 5%, heated to 98°C for 10 minutes, and electrophoresed though replicate discontinuous SDS-polyacrylamide gels (5% stacking, pH 6.8, 12% resolving, pH 8.8). After electrophoresis, one gel was rinsed in distilled H_2O, and electroblotted to a PVDF membrane (MSI, Westboro, MA), while the other was stained with Coomassie Brilliant Blue to verify identical lane loading. After the transfer, the blot was blocked for 1-2 hours in TBS-Tween (TBST, 100 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 5% nonfat dry milk. The blot was incubated for 1 hour with a 1:1000 dilution (in blocking solution) of the CR-1 polyclonal antiserum (CR-67), washed 3 × 10 minutes in TBST, incubated for 1 hour in a 1:3000 dilution of goat-anti-rabbit-alkaline phosphatase antibody (Bio-Rad, Hercules, CA), washed 3 × 10 minutes in TBST, and incubated for 5 minutes with the CDP-Star chemiluminescent substrate (New England Biolabs, Beverly, MA). Bands were detected by exposure of x-ray film.

RT-PCR. Total RNA was isolated from the -SA/pBKCMV control clone #14, -SA/pRZECR-1 clones #4, 5, 11, 18, 23, and 24, -SA cells, and +SA cells using Ultraspec RNA Isolation Reagent (Biotecx, Houston, TX). mRNA (polyA$^+$) was isolated from the total RNA preps using oligo dT/magnetic bead separation (PolyATtract, Promega, Madison, WI). Approximately 1 ng of polyA$^+$ mRNA was reverse transcribed with MoMuLV RT (Promega, Madison, WI), and 1/10 of each RT reaction was amplified using *Taq* polymerase (Promega, Madison, WI) and the following primers (fwd, CCCATCCCCTGCCGGTCT; rev, AAGCGAGGCGCCAGCTAG); this generated the expected fragment of 716 bp. Control reactions (1/10 of each RT reaction) using actin primers generated the expected 360 bp fragment. RT-PCR reactions were electrophoresed through 1.5% agarose-TAE gels, stained with ethidium bromide and photographed. The photographs were scanned and then analyzed for band intensities (Intelligent Quantifier, BioImage, Ann Arbor, MI).

RESULTS

Construction of the CR-1-Specific Ribozyme

Using the published sequence of mouse CR-1 (2), we designed a hammerhead ribozyme (13,14) which recognizes nucleotides 12-28 of the murine CR-1 mRNA and should cut after the GUC triplet at nucleotides 18-20. This site was chosen for three reasons. First, an RNA folding analysis (mfold, by Zuker and Jaeger, using the GCG Wisconsin Software package) of the first 1050 bases of the mRNA showed that this site should be accessible. Specifically, residues 7-17 and 22-25 form single-stranded loops in the 14 best structure predictions. Second, this site is at the extreme 5′ end of the mRNA, and cleavage or binding at this site will thus result in the immediate elimination of CR-1 translation. Cleavage further along the message might allow production of a truncated, but still bioactive, peptide prior to degradation of the message. Finally, it has been shown that stem-loops engineered into an RNA sequence within 12 nt of the 5′ cap can prevent the 40S ribosomal subunit from binding to the mRNA (15). Binding of the ribozyme at this site may provide secondary structure that will inhibit binding of the 40S subunit, even if the mRNA is not cleaved. The 39-nucleotide hammerhead molecule was synthesized as two complementary 43-nucleotide strands with 5′ overhangs. These overhangs allowed for efficient directional cloning into the pBKCMV eukaryotic expression vector. Proper insertion was determined by restriction analysis (data not shown) and verified by sequencing. A diagram of the CR-1 message and ribozyme is shown in Figure 1A, and the folded hammerhead, bound to the CR-1 substrate mRNA is shown in Figure 1B.

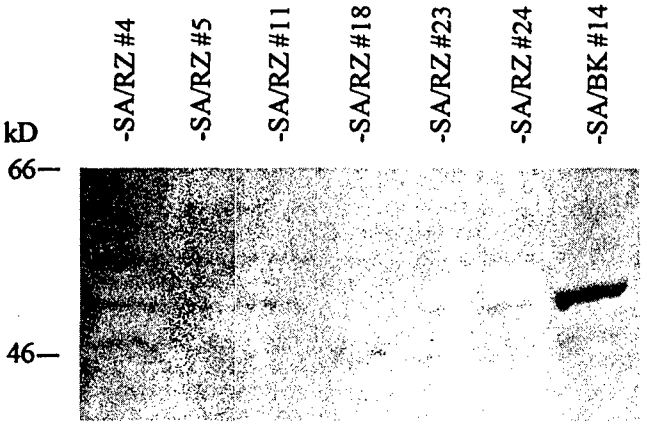
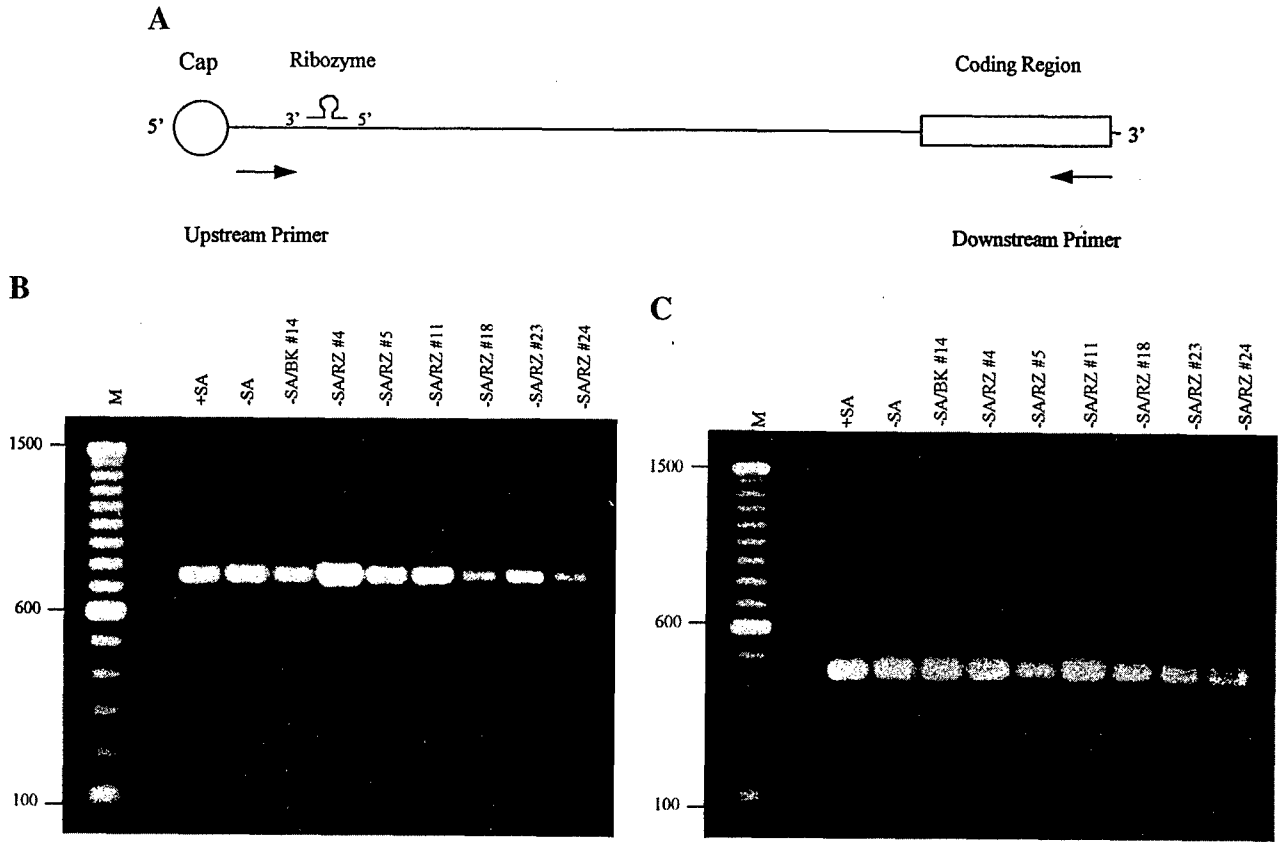


FIG. 2. Western blot of -SA clones transfected with pBKCMV or pRZECR-1. Lane kD, molecular weight in kilodaltons; 2-6, -SA clones transfected with pRZECR-1; 7, -SA control clone transfected with pBKCMV.

Testing of the Ribozyme

The control expression vector (pBKCMV) and the ribozyme expression vector (pRZECR-1) were transfected into -SA cells using Lipofectamine. Bulk cultures were selected for the presence of the vectors with G418 (Geneticin), and at least 12 stably-transfected clones were picked from each transfection. For each transfection, protein was harvested from twelve clones and CR-1 expression was tested by western blot using the CR-67 antiserum. Figure 2 shows one -SA/BKCMV control clone and 6 -SA/RZECR-1 clones. -SA/RZECR-1 clones # 5, 18, and



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Cell Line	Lane	CR-1	Actin	CR-1/ Actin
+SA	3	8.92	10.23	.87
-SA	4	9.07	10.33	.88
-SA/pBKCMV #14	6	6.56	10.54	.62
-SA/pRZECR-1 #4	7	12.66	9.78	1.30
-SA/pRZECR-1 #5	8	8.56	6.37	1.34
-SA/pRZECR-1 #11	9	9.77	10.53	.93
-SA/pRZECR-1 #18	10	3.30	7.20	.46
-SA/pRZECR-1 #23	11	6.10	6.54	.93
-SA/pRZECR-1 #24	12	2.31	5.63	.41

FIG. 3. RT-PCR Analysis of mRNA expression in transfected -SA clones. (A) Diagram of the 5′ portion of the CR-1 mRNA, showing the 5′ cap, ribozyme binding site, coding region, and PCR primers. (B) Electrophoretic analysis of RT-PCR using the CR-1-specific PCR primers. Lane 1, 100 bp marker; 2, blank; 3-4, untransfected controls; 5, -SA control clone transfected with pBKCMV; 6-11, -SA clones transfected with pRZECR-1. (C) Electrophoretic analysis of RT-PCR using actin-specific PCR primers. Lane 1, 100 bp marker; 2, blank; 3-4, untransfected controls; 5, -SA control clone transfected with pBKCMV; 6-11, -SA clones transfected with pRZECR-1. (D) Relative intensities of RT-PCR Bands in panels B and C.

23 show complete loss of expression of the CR-1 protein, and -SA/RZECR-1 clones # 4, 11, and 24 show near-complete loss of expression. All -SA/pBKCMV clones showed similar levels of CR-1 expression (data not shown).

Mechanism of Activity of the Ribozyme

It was apparent from the western blot that the ribozyme was effective at eliminating CR-1 expression, but

not whether this was through an enzymatic or other mechanism. The cleavage site for the message was too near the 5′ end of the CR-1 message to distinguish cleaved from non-cleaved message on a Northern blot. Therefore we opted for a reverse transcriptase-PCR strategy. Primers were designed that spanned the ribozyme cut site and would only amplify a message that had not been cleaved by the ribozyme (Figure 3A). As shown in Figure 3B, all clones make uncleaved CR-1 message. As shown in Figure 3C, most reactions show similar levels (less than 2-fold difference between lanes) of actin transcript. -SA/RZECR-1 clones #5, #18, #23, and #24 show slightly lower levels of actin, but these variations are smaller than those seen with CR-1 (up to 5.5-fold difference between lanes). After analyzing the bands densitometrically (Figure 3D), and normalizing the CR-1 levels to the actin ones, it appears that there are reduced levels of CR-1 message in clones -SA/pRZECR-1 #18 and 24. Amplification of polyA$^+$ RNA samples without reverse transcription did not yield any fragments (data not shown). Given the data in Figures 2 and 3, it appears most likely that this hammerhead ribozyme is reducing the levels of CR-1 protein primarily through a non-cleavage mechanism.

DISCUSSION

While both antisense RNA and ribozymes can effectively reduce expression of target genes (for example (10,11,14,16-19)), we originally chose to develop a ribozyme because of its catalytic nature. Antisense oligonucleotides must generally be produced in stoichiometric amounts to eliminate synthesis of the target protein (20), while a single ribozyme molecule may cleave multiple copies of the target RNA (21,22).

We have now demonstrated that a hammerhead molecule specific for the extreme 5′ region of the CR-1 mRNA greatly reduces the expression of CR-1 in a tumorigenic mouse mammary epithelial cell line. This construct has excellent activity. However, the apparent method of action of this molecule requires some consideration. The first question that must be addressed is the apparent lack of cleavage activity. Many studies have shown that the length of the ribozyme and target molecules can have dramatic effects on the efficiency of cleavage (see (23, 24) for an example of each). The expression construct used in this system produces a ribozyme transcript of approximately 800 nt, only 40 of which constitute the ribozyme itself. These extraneous sequences may result in altered folding or binding which inhibit cleavage activity.

The second question to be addressed is how this molecule is so effective at eliminating CR-1 protein expression. Based on an assessment of the 5′ untranslated region sequence, CR-1 may be translationally regulated in its native state. It has 2 upstream open reading frames (2), the first of which has a start codon in optimal context (25). In addition, CR-1 appears to act as a transforming growth factor, and many of these types of molecules exhibit translational control of expression (26). The ribozyme, a relatively large (800nt) molecule, binds very near the 5′ cap, close enough to inhibit binding of the 40S ribosomal subunit (15). This circumstance may very efficiently block translation, regardless of the ability of the ribozyme to cleave its target.

There is evidence for decreased message levels in some of the ribozyme-transfected clones, and it is possible that this ribozyme acts through mRNA cleavage as well. However, there is no correlation between the apparent degree of cleavage activity and level of protein synthesis suppression. The two clones with the lowest CR-1/actin ratios (18 and 24) come from both the group with nearly complete and the group with complete elimination of CR-1 protein expression. Therefore, it appears that inhibition of translation is the more important mechanism.

In summary, we have shown that a hammerhead ribozyme directed toward the extreme 5′ end of the murine CR-1 message is effective at reducing CR-1 protein levels in a tumorigenic murine mammary epithelial cell line. This ribozyme appears to act principally through a non-enzymatic mechanism, though it may also cleave the mRNA in a classic ribozyme fashion. It is highly effective at eliminating CR-1 protein expression, perhaps due to acting through both inhibition of translation and cleavage mechanisms. In general, the targeting of ribozyme-like molecules to the extreme 5′ ends of mRNAs may be a very effective method of reducing protein expression in part by reducing the ability of the 40S ribosomal subunit to bind the target message.

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REFERENCES

1. Ciccodicola, A., Dono, R., Obici, S., Simeone, A., Zollo, M., and Persico, M. G. (1989) *EMBO J.* **8,** 1987–1991.
2. Dono, R., Scalera, L., Pacifico, F., Acampora, D., Persico, M. G., and Simeone, A. (1993) *Development* **118,** 1157–1168.
3. Ciardiello, F., Kim, N., Saeki, T., Dono, R., Persico, M. G., Plowman, G. D., Garrigues, J., Radke, S., Todaro, G. J., and Salomon, D. S. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7792–7796.
4. Saeki, T., Stromberg, K., Qi, C.-F., Gullick, W. J., Tahara, E., Normanno, N., Ciardiello, F., Kenney, N., Johnson, G. R., and Salomon, D. S. (1992) *Cancer Res.* **52,** 3467–3473.
5. Qi, C.-F., Liscia, D. S., Normanno, N., Merlo, G., Johnson, G. R., Gullick, W. J., Ciardiello, F., Saeki, T., Brandt, R., Kim, N., Kenney, N., and Salomon, D. S. (1994) *Br. J. Cancer* **69,** 903–910.
6. Saeki, T., Salomon, D., Normanno, N., Johnson, G. R., Gullick, W. J., Mandai, K., Moriwaki, S., Takashima, S., Kuniyasu, M.,

Tahara, E., Kawami, H., Nishiyama, M., and Toge, T. (1994) *Int. J. Oncol.* **5,** 215–223.

7. Friess, H., Yamanaka, Y., Büchler, M., Kobrin, M. S., Tahara, E., and Korc, M. (1994) *Int. J. Cancer* **56,** 668–674.
8. Ciardiello, F., Dono, R., Kim, N., Persico, M. G., and Salomon, D. S. (1991) *Cancer Res.* **51,** 1051–1054.
9. Brandt, R., Normanno, N., Gullick, W. J., Lin, J.-H., Harkins, R., Schneider, D., Wallace-Jones, B., Ciardiello, F., Persico, M. G., Armenante, F., Kim, N., and Salomon, D. S. (1994) *J. Biol. Chem.* **269,** 17320–17328.
10. Ciardiello, F., Tortora, G., Bianco, C., Selvam, M. P., Basolo, F., Fontanini, G., Pacifico, F., Normanno, N., Brandt, R., Persico, M. G., Salomon, D. S., and Bianco, A. R. (1994) *Oncogene* **9,** 291–298.
11. Baldassarre, G., Bianco, C., Tortora, G., Ruggiero, A., Moaser, M., Dmitrovsky, E., Bianco, A. R., and Ciardiello, F. (1996) *Int. J. Cancer* **66,** 538–543.
12. Danielson, K. G., Anderson, L. W., and Hosick, H. L. (1980) *Cancer Res.* **40,** 1812–1819.
13. Forster, A. C., and Symons, R. H. (1987) *Cell* **49,** 211–220.
14. Sullivan, S. M. (1994) *J. Invest. Dermatol.* **103,** 85S-89S.
15. Kozak, M. (1989) *Mol. Cell. Biol.* **9,** 5134–5142.
16. Moroni, M. C., Willingham, M. C., and Beguinot, L. (1992) *J. Biol. Chem.* **267,** 2714–2722.
17. Ciardiello, F., Bianco, C., Normanno, N., Baldassarre, G., Pepe, S., Tortora, G., Bianco, A. R., and Salomon, D. S. (1993) *Int. J. Cancer* **54,** 952–958.
18. Shore, S. K., Nabissa, P. M., and Reddy, E. P. (1993) *Oncogene* **8,** 3183–3188.
19. Kashani-Sabet, M., Funato, T., Florenes, V. A., Fodstad, O., and Scanlon, K. J. (1994) *Cancer Res.* **54,** 900–902.
20. Denhardt, D. T. (1992) *Ann. N. Y. Acad. Sci.* **660,** 70–76.
21. Uhlenbeck, O. C. (1987) *Nature* **328,** 596–600.
22. Cech, T. R., and Uhlenbeck, O. C. (1994) *Nature* **372,** 39–40.
23. Ohkawa, J., Yuyama, N., Takebe, Y., Nishikawa, S., and Taira, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11302–11306.
24. Campbell, T. B., McDonald, C. K., and Hagen, M. (1997) *Nucleic Acids Res.* **25,** 4985–4993.
25. Kozak, M. (1991) *J. Biol. Chem.* **266,** 19867–19870.
26. Kozak, M. (1991) *J. Cell Biol.* **115,** 887–903.